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DIFFERENTIAL REGULATION OF ENDOTHELIUM BEHAVIOUR BY PROGESTERONE AND MEDROXYPROGESTERONE ACETATE

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ABSTRACT

Medroxyprogesterone acetate (MPA) is a synthetic progestin commonly used in hormone replacement therapy (HRT). The aim of this work was to study and compare the effect of progesterone (Pg) and MPA, on the regulation of cellular events associated with vascular homeostasis and disease. Platelet adhesion to endothelial cells (ECs), nitric oxide (NO) production, and cell migration were studied using murine endothelial cells in vitro exposed to the progestins. After seven minutes treatment, MPA significantly inhibited NO synthesis with respect to control value; meanwhile Pg markedly increased vasoactive production. In senile ECs, the stimulatory action of Pg decreases; meanwhile MPA maintained its ability to inhibit NO synthesis.The presence of RU486 antagonized each steroid action. When ECs were preincubated with PD98059 (MAPK inhibitor) or chelerythrine (PKC inhibitor) before Pg or MPA treatment, the former totally suppressed the steroid action, but the PKC antagonist did not affect NO production. In the presence of a PI3K inhibitor (LY294002) a partial reduction in Pg effect, and a reversal of MPA action was detected. Using indomethacin the contribution of cyclooxygenase (COX) pathway was also detected. On platelet adhesion assays, Pg inhibited and MPA stimulated platelet adhesion to ECs. Under inflammatory conditions, Pg prevented platelet adhesion induced by lipopolysaccharide (LPS); meanwhile MPA potentiated the stimulatory action of LPS. Finally, although both steroids enhanced ECs migration, MPA exhibited a greater effect. In conclusion the data presented in this research provide evidence of a differential regulation of vascular function by Pg and MPA.

Keywords: cell migration**,** medroxyprogesterone acetate**,** nitric oxide**,** progesterone**,** vascular tissue

INTRODUCTION

The endothelium is the main regulator of vascular physiology that contributes to maintain vascular tone and non thrombogenic properties of endothelial surface. Endothelial NO is key factor of vascular homeostasis with antiatherogenic and antithrombotic properties. NO is a potent vasodilator which prevents platelet aggregation (Ignarro 1989) and leukocytes adhesion to the endothelium (Tsao *et al.* 1995), and decreases the expression of proinflammatory genes (Harrison *et al.* 2006). Several cardiovascular risk factors such as oxidative stress, alterations in plasmatic lipid profile, hypertension, viral infections, and diabetes mellitus, promote endothelial dysfunction and consequently vascular diseases, mainly atherosclerosis. The genesis of atheromatous lesions has a multifactorial pattern characterized by impaired synthesis of vasoactive molecules (NO and prostacyclin), enhanced synthesis and expression of cell adhesion molecules (CAMs) involved in leukocyte infiltration and platelet adhesion, as well as an altered profile of cell proliferation and migration. The initial response to vascular injury includes activation, adhesion, and aggregation of platelets to endothelium, events that release cytokines and enhance leukocyte recruitment (Ross 1999). In vascular diseases, ECs turnover is altered (Choy *et al.* 2001). After endothelium damage, vessel repair is achieved by migration and proliferation of adjacent ECs (Cartwright *et al.* 2000; Moreno *et al.* 2009).

Menopause hypoestrogenism represents a physiological risk factor for cardiovascular disease, fact attributed to the loss of 17β-estradiol (E2) cardioprotection. However, beside E2 circulating levels diminution, during menopause the ovarian synthesis of Pg is also affected. HRT based on estrogen, or combined with natural or synthetic progestins like MPA, emerges as an alternative to relieve the signs and symptoms associated with menopause. Although the large body of information derived from in vitro experimental studies that confer to E2 a relevant cardioprotective action, this cannot be translated into health benefit when HRT is applied to postmenopausal women. This raises the controversy about the risk/benefit of HRT on cardiovascular disease. Historically, progestins were included in the HRT protocols to counteract the endometrial dysplasia caused by E2. It has been reported that administration of MPA improves the effectiveness of HRT (Manson *et al.* 2003). However, other studies have shown that co-administration of progestins

blocks the atheroprotective effect of estrogen (Sitruk-Ware 2000). The secondary cardiovascular prevention study HERS (Heart and Estrogen/progestin Replacement Study) showed that HRT performed by oral administration of conjugated equine estrogen (CEE) and MPA for four years, was not able to reduce the incidence of coronary events, and in turn, increased the risk of thromboembolic events in postmenopausal women with established coronary artery disease (Grady *et al.* 2002; Hulley *et al.* 1998). Indeed, in the Women's Health Initiative (WHI) trial, CEE plus MPA administration to healthy postmenopausal women resulted in a substantial increase in risk of myocardial infarction (Rossouw *et al.* 2002). In contrast, another study also performed in healthy postmenopausal women showed that oral administration of E2 plus Pg, improved circulating levels of coagulation factors and vascular health (van Baal *et al.* 1999).

Vascular function is regulated by several hormones and factors including sex steroids. Both, ECs and vascular smooth muscle cells (VSMCs) possess estrogen receptor and Pg receptor (PgR) (Joswig *et al.* 1999; Vazquez *et al.* 1999). Current knowledge about the biochemical action of natural Pg and other progestins at vascular level is quite low in contrast to the large amount of evidence reported to E2. In the last years, potential vascular effects of Pg have been described. In rabbit coronary arteries and rat aorta, Pg induces endothelium-independent vasorelaxation (Jiang *et al.* 1992). Moreover, Pg regulates vascular cells growth (Glusa *et al.* 1997; Simoncini *et al.* 2003) and inhibits adhesion molecules expression induced by tumour necrosis factor-alpha (TNF-α) and LPS (Glusa *et al.* 1997; Otsuki *et al.* 2001; Simoncini *et al.* 2004). We have previously demonstrated that in rat aortic tissue, Pg non genomically induces nitric oxide synthase (NOS) and COX stimulation through a mechanism of action that involves mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling pathways, and a cross-talk between NOS and COX systems. The rapid action of the steroid also includes an inhibition of platelet aggregation dependent on NOS and phospholipase C (PLC) transductions systems (Mendiberri *et al.* 2006). The fast effects elicited by the hormone are required for the regulation of ECs growth (Cutini *et al.* 2009). Pg also enhances cell proliferation, migration, and apoptosis of VSMCs. The regulation of muscle cell growth matched with the two step model of steroid action that integrates genomic and non genomic actions (Cutini *et al.* 2009; Cutini & Massheimer 2010).

There is evidence in the literature that Pg and MPA are not equivalent in terms of molecular signaling in human and murine vascular tissue. It has been reported that Pg and MPA activate different signaling events *in vitro* (L'hermite *et al.* 2008). MPA and other synthetic progestins exert different inflammatory and anti-inflammatory effects *in vitro* when compared with Pg (Simoncini *et al.* 2004). Therefore, the aim of this work was to study and compare the effect of Pg and MPA on the regulation of cellular events associated with vascular homeostasis and disease: platelet adhesion to ECs, NO production, and cell migration.

MATERIALS AND METHODS

Materials

Pg was obtained from Calbiochem-Novabiochem International (San Diego, CA, USA). Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10×), l-glutamine (100×), amphotericin B (0.25 mg/mL), penicillin/streptomycin (100×), and fetal calf serum were obtained from PAA Laboratories (Pasching, Austria). Dulbecco's modified Eagle's medium (DMEM), MPA, N-nitro-L-arginine methyl ester (L-NAME), and all other reagents were purchased from Sigma Chemical Company (St Louis, MO, USA).

Animals

Young (3–5 weeks old) and old (24 months old) female Wistar rats were employed. They were fed with standard rat food, given water *ad libitum* and maintained on a 12 h light/12 h dark cycle. All the procedures were performed in accordance with the guidelines published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.* All procedures involving animal and their care were performed at the Unit of Animal Care belonging to the Department of Biology, Biochemistry and Pharmacy at the University. The Animal Care Use Committee of this Unit approved the protocol used.

The absence of ovarian activity in old female rats was performed by daily controls of oestrous cycle activity evaluated by optical microscope examination of vaginal smears. No estrous cycles were detected for at least 2-4 consecutive months (Selles *et al.* 2005).

ECs culture

ECs cultures were obtained from aortic rings explants isolated from young Wistar female rats (3–5 weeks old) (Yeh *et al.* 2002). When ECs of senile rats (senile ECs) were employed, aortic rings explants were isolated from aged Wistar rats (1.5–2 years old). Briefly, animals were killed by cervical dislocation and the full length thoracic aorta was aseptically removed. Immediately after, the aorta was cleaned of adherent connective tissue, and cut into small ring-shaped segments. Ring explants were seeded in a 60mm matrix-coated Petri dishes (NUNC) containing phenol red-free DMEM supplemented with 20% (v/v) fetal calf serum (FCS), 60 µg/mL penicillin, 2.5 µg/mL amphotericin-B, 2 mM L-glutamine, and 1.7 g/L sodium bicarbonate. Explants were incubated at 37ºC in 5% CO_2 atmosphere. In order to establish a pure ECs culture, after three days of culture the ring explants were removed and ECs were allowed to reach confluence. The identity of the ECs was determined (a) by phase-contrast microscope observation of the characteristic morphology of cobblestone shape growth in confluent monolayer, (b) by positive immunocytochemistry reactivity to Factor VIII and to anti-Vimentin, clone V9 using DakoCytomation EnVision system, and (c) by the bioability to synthesize NO (Campelo *et al.* 2012). Cells from passages 2–7 were used for all experiments. Fresh medium containing 10% (v/v) FCS was replaced every 72 h. Steroid solutions employed in the cellular treatments were prepared using isopropanol as solvent. The final concentration of the vehicle was always below than 0.1% (v/v).

Hematoxylin and Eosin staining

ECs were grown in 35 mm culture dishes (NUNC). Confluent ECs were washed twice with phosphate-buffered saline (PBS) and fixed with glutaraldehyde 0.1% (v/v). The cells were stained with hematoxylin and eosin solution. Images were recorded using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51).

Immunofluorescence detection of vascular endothelial growth factor receptor 2 (VEGFR2) and CD34 expression

ECs were grown in 96-well optical bottom plates (NUNC). Confluent ECs were washed twice with PBS and fixed with paraformaldehyde 2% (v/v) in PBS for 15 minutes. Then cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 minutes, and incubated with 1% albumin for 1 hour to prevent non-specific antibody binding. Cells were immunostained with anti-CD34 (8G12 BD Biosciences) or anti-VEGFR2 (FAB357P BD Biosciences). Cell nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Immunofluorescence was visualized using an Olympus BX51 microscope equipped with a Q-Color 3 digital camera (Olympus America, Center Valley, PA).

Platelet assays

Platelet isolation

For platelet aggregation assays, rat platelet-rich plasma (PRP) was obtained as previously described (Cutini *et al.* 2012). For platelet adhesion assays, PRP was fixed in 4% (v/v) formaldehyde/PBS for 10 minutes, suspended in PBS (pH 7.4) at room temperature and centrifugated at 750 × g for 10 minutes. Then, fixed platelets were washed twice with PBS. Manual platelet count was done using a hemacytometer. Finally, platelets were suspended at a final concentration of 5×10^6 platelets/mL in DMEM supplemented with 1% (v/v) FCS.

Platelet adhesion assay

Confluent monolayers of ECs seeded on 24-multiwell culture plates (NUNC) were starved for 24 h with serum-free DMEM, and then exposed to Pg, MPA, LPS (1 µg/mL) or steroids plus LPS in DMEM supplemented with 1% (v/v) FCS. Once finished treatment, ECs were washed two times with PBS, and 300 µL of platelet suspension were added on pretreated cells. Platelets were allowed to adhere to ECs for 2 h at 37ºC. Supernatants of each well containing non-adhered platelets were collected and counted employing a hemacytometer. The number of adhered platelets to ECs was calculated by difference between total added platelets and non-adherent platelets. Results are expressed as mean ± SD of the number of adhered platelets/µL.

Platelet aggregation assay

Platelet aggregation was measured using a turbidimetric assay as previously described (Cutini *et al.* 2012). ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% (v/v) FCS and allowed to grow to 60–70% of confluence. Culture medium was replaced by 400 µl of PRP, 3×10^8 platelets/mL) and exposed to 10 nM Pg, 10 nM MPA or vehicle (control) for 5 minutes. Immediately after treatment, 285 µl of PRP was taken and set in a CronoLog 430 aggregometer cuvette with continuous stirring. Aggregation was initiated by the addition of 2×10^{-5} M adenosine diphosphate (ADP). Control group containing ECs in PRP was treated with vehicle alone (isopropanol <0.1%, v/v). Changes in light transmission were recorded for 5 minutes after ADP addition. The signal generated by a platelet-poor plasma was taken as 100% transparent control (Kikuta *et al.* 1998). ECs were dissolved in 1 M NaOH and aliquots were taken for protein determination by Lowry's method. Results were expressed as percentage of inhibition of platelet aggregation with respect to control. The maximal platelet aggregation was considered to be that induced by the control samples. Two animals were used for each experiment. When the direct effect of the progestins on platelets were evaluated, PRP was incubated with the Pg or MPA in the absence of ECs, and platelet aggregation was measured as described above. Basal aggregation was considered the maximal aggregation exhibited by PRP alone, without vehicle or steroid treatment. Results were expressed as percentage of platelet aggregation with respect to basal.

Measurement of NO production

ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3.5×10^4 cells/well and allowed to grow to 90% of confluence in DMEM containing 10% (v/v) FCS. Steroid treatment was performed in fresh DMEM containing 1% (v/v) FCS by addition of Pg or MPA for 7 minutes. Respective controls (vehicle alone) were also processed. When specific antagonist/inhibitors were used, they were added 30 minutes before steroid treatment. Nitrites (NO_2^-) were measured in the incubation media as a stable and non-volatile breakdown product of the NO released, employing the spectrometric Griess reaction (Campelo *et al.* 2012). Briefly, once finished treatment, aliquots of culture medium supernatant were mixed with Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated 10 minutes at room

temperature. Absorbance was measured at 548 nm in a microplate reader (Biotek Synergy-HT). The concentration of NO_2^- in the samples was determined with reference to a sodium nitrite ($NaNO_2$) standard curve performed in the same matrix. Cells were dissolved in 1 M NaOH, and protein content was measured by Lowry Method (Lowry *et al.* 1951). The results were expressed as nmol of NO_2^- per mg protein.

Cell migration assay

Cells were seeded at a density of 5×10^5 cells/cm^2 in 60mm NUNC dishes with DMEM containing 10% (v/v) FCS, and were grown to 90% confluence. Cells were starved for 24 h with serum-free medium. In order to evaluate cell migration, a wound was made by pressing a razor blade down on the dish and remove part of the monolayer (Pedram *et al.* 2002). Immediately after, adhered cells were washed twice with PBS and cultured in fresh DMEM containing 1% (v/v) FCS plus Pg, MPA or vehicle control. After 48 h of culture, cells were fixed with glutaraldehyde 0.1% (v/v) and stained with Giemsa solution. Migration was quantified by counting the number of cell nuclei that migrated at scratched area in at least seven different representative fields of each culture plate. Cell migration was recorded using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51). Results are expressed as mean ± SD of the number of migrated cells/field.

Crystal Violet assay

Endothelial cells were seeded on 96-multi-well plates (NUNC) at a density of 5×10^3 cells/well in DMEM supplemented with 10% (v/v) FCS and allowed to grow to 60–70% confluence. Cells were synchronized by placing in serum-free DMEM for 24 h, and further exposed to different concentrations of Pg, MPA or vehicle (control) for 24 h in fresh DMEM containing 1% (v/v) FCS. Medium was removed, the cells were fixed in 1% glutaraldehyde for 15 minutes and subsequently stained for 30 minutes in 0.1% crystal violet. Immediately after, the cells were washed twice, air-dried, and dissolved in 0.2 % triton. Absorbance of each well was measured at 590 nm in a microplate reader (Biotek Synergy-HT). Cell viability was determined as optical density (OD)

increase, and maximal viability was considered the OD registered from control group. Results are expressed as percentage of cell viability with respect to control.

Statistical analysis

The results presented were obtained from three independent experiments where each individual experimental condition was performed by quadruplicate (n=4). All data are presented as mean ± SD. Different cell cultures were used for each independent experiment. Comparisons between two means were made using Student's t-test, and multiple comparisons with one or two ways ANOVA, followed by Fisher's least significant difference test, using SSPS Statistical software for Windows. P-values lower than 0.05 were considered to be statistically significant.

RESULTS

Having in mind our previous reports in rat aortic tissue concerning the non-genomic action of Pg on NO synthesis (Cutini *et al.* 2009; Mendiberri *et al.* 2006; Selles *et al.* 2002), using isolated ECs we studied the rapid regulation of NO production by different concentrations of Pg and MPA. To that end, cells were exposed to Pg or MPA for 7 minutes. Fig. 1A shows that MPA treatment significantly inhibited NO synthesis with respect to control value (43, 40, and 49% below control; 1, 10, and 100 nM MPA respectively; $P<0.02$). On the other hand, when ECs were treated with the same concentrations of Pg, a marked stimulus in the vasoactive synthesis was detected (41, 64, and 96% above control; 1, 10, and 100 nM Pg respectively; $P<0.02$) (Fig. 1A). In order to determine whether Pg and MPA were affecting NOS derived NO, we measured the rapid production of NO in the presence of an irreversible NOS inhibitor (L-NAME). As shown in Fig. 1A, 10 μM L-NAME, completely suppressed the effects evoked by the progestogens.

The effect of Pg and MPA on ECs viability was evaluated using crystal violet assay. The results presented in Table 1 show that maximal cell viability was observed in all experimental conditions, ruling out a negative effect of progestins on cell survival.

The effects of Pg and MPA were also tested in ECs isolated from senile rats devoid of ovarian activity. The stimulatory action of Pg markedly decreased; meanwhile MPA maintained its ability to inhibit NO synthesis (Fig. 1B). Negative controls with L-NAME were also performed. We also

checked the existence of phenotypic differences between young and senile derived ECs. Fig. 2 shows that, similar morphological features (hematoxylin and eosin staining) and comparable ECs markers expression (VEGFR2; CD34) were found in both groups.

In order to evaluate the participation of PgR on the steroids effect on NO production, we employed an antagonist of PgR, the compound RU486. ECs were preincubated 30 minutes with 1 μM RU486 prior to steroid treatment. We obtained evidence that the antagonist affected either the fast stimulatory or the inhibitory action on NO production exerted by Pg or MPA respectively (Fig. 3). The presence of the PgR antagonist partially suppressed the stimulatory action of Pg on NO synthesis (64 and 96% vs 35 and 47% above control, Pg 10 and 100 nM in the absence vs presence of RU486, respectively, $P<0.05$) (Fig. 3). Similar results were obtained when the cells were treated with 1 nM Pg or 1 nM MPA (data not shown). Simultaneous preincubation of ECs with RU486 and L-NAME prior to hormonal treatment, blunted NO production (Fig. 3). We also evaluate the probable participation of androgen receptor (AR) using flutamide as AR antagonist. The presence of flutamide did not affect progestins action on NO synthesis (data not shown).

The involvement of protein kinase C (PKC), MAPK, and PI3K intracellular signaling transduction pathways, on Pg and MPA regulation of vasoactive production was investigated. To that end, ECs were preincubated 30 minutes in the absence or presence of LY294002 (PI3K inhibitor), PD98059 (MAPK inhibitor) or chelerythrine (PKC inhibitor) before treatment with Pg or MPA.

The MEK (mitogen-activated protein kinase kinase) inhibitor (PD98059) totally abolished the stimulatory action on NO synthesis elicited by 10 nM Pg (Fig. 4A). On the other hand, the inhibition of PKC signaling pathway with chelerythrine did not modify the stimulation on NO production induced by the natural progestogen. Meantime, a partial reduction in Pg effect was observed in the presence of LY294002 (Fig. 4A). Similar results were obtained when hormonal treatments were performed with 1 or 100 nM Pg, or in the presence of other PI3K inhibitor (wortmannin) (data not shown).

As we can be seen in Fig. 4B, the presence of PD98059 or LY294002 completely suppressed the inhibitory effect on NO production exerted by 10 nM MPA treatment. Noteworthy that, the presence of the PI3K pathway inhibitor, not only completely abolished the inhibitory effect of MPA on vasoactive synthesis but also enhanced NO production with respect to control. As observed with

Pg treatment, when ECs were pretreated with the PKC inhibitor, the action of 10 nM MPA was not modified (Fig. 4B). Similar results were obtained when ECs treatment was performed with 1 or 100 nM MPA (data not shown). Again, the negative control performed using L-NAME confirmed the progestin's actions on NOS derived NO (Fig. 4A and 4B).

Having in mind that our previous work demonstrated that in rat aortic strips Pg mechanism of action is dependent on a cross talk between COX and NOS pathways that involves COX stimulation for NOS synthesis (Selles *et al.* 2002), we performed additional experiments to test whether, in isolated ECs the effect of progestins on NO production is dependent on COX activity. To that end, indomethacin was used as COX inhibitor. As can be observed in Fig. 5 pretreatment with indomethacin reversed the effect of Pg or MPA on NO synthesis.

Since NOS and COX end products can affect platelet function, In order to study whether the steroids were able to regulate platelet-ECs interactions, platelet adhesion and platelet aggregation assays were performed. Cells were incubated with Pg or MPA for 24 h in the presence or absence of a platelet adhesion inductor, bacterial LPS, which was added during the last 21 h of steroid treatment. Once treatment was finished, an exact number of platelets was seeded on ECs monolayers and allowed to adhere for 2 h. Fig. 6A shows that treatment with Pg (1, 10, and 100 nM) significantly inhibited platelet adhesion to ECs with respect to the control (29, 26, and 22% below control; Pg 1, 10, and 100 nM respectively; $P<0.05$). On the other hand, when cells were exposed to LPS, platelet adhesion was markedly increased (69% above control, $P<0.001$). However, pre-treatment of ECs with 1, 10, and 100 nM Pg, totally abolished platelet adhesion induced by the proinflammatory agent (Fig. 6A). By contrast, treatment of cells with 1, 10, and 100 nM MPA significantly increased platelet adhesion (33, 37, and 44% above control; MPA 1, 10, and 100 nM respectively; $P<0.05$) (Fig. 6B). Moreover, we can see that, unlike Pg results, pre-treatment of cells with MPA, potentiated the stimulatory action of LPS on platelet adhesion (Fig. 6B).

To test the effect of the progestins on platelet aggregation, ECs were incubated with PRP and subsequently exposed to Pg or MPA for 5 minutes. Immediately, aliquots of PRP were transferred to the aggregometer and ADP-induced platelet aggregation was measured. Table 2 shows the quantitative data obtained. Similarly as our previous data obtained with rat aortic strips (Sellés *et*

al, 2002), Pg exhibited a markedly antiaggregatory action, meanwhile the inhibition of platelet aggregation (IPA) induced by MPA was lower (82.3 vs 27.2% IPA with respect to control, Pg vs MPA). A direct action of the progestins on platelets was ruled out, since the addition of Pg or MPA to PRP in the absence of ECs, induced maximal aggregation similar to basal group (Table 2). Afterwards, using wound healing assays, we examined the effect of Pg and MPA on ECs migration. Fig. 7 shows that 48 h treatment with Pg induced a significant enhance in the number of cells that cross the wound border and migrated to removed area in a wide range of concentrations tested (5-100 nM). At lower doses (1 nM Pg) no significant differences were detected in the number of migrant cells with respect to the control group (Fig. 7).

When the synthetic progestin was assayed, the evidence shows that MPA also increased cell migration to denuded area compared to non-treated cells at all concentrations tested (Fig. 8). The magnitude of the incremental increase induced by MPA was much higher than the stimulatory action elicited by Pg (1168% vs 275%; 10 nM MPA vs 10 nM Pg respectively, $P<0.001$) (Fig. 7 and Fig. 8).

Finally, Fig. 9 shows a schematic diagram that summarise the results obtained.

DISCUSSION

The data presented in this research provide evidence of a differential regulation of vascular function by Pg and MPA. Pg markedly stimulates NO production; meanwhile MPA inhibits its synthesis. Although Pg and MPA exhibit divergent effects on the vasoactive generation, both mechanisms of action involve at least in part, the participation of PgR; COX; PI3K; and MAPK signal transduction pathways. Under inflammatory conditions Pg prevents platelet adhesion to ECs. In contrast, MPA potentiates platelet adhesion. Both steroids enhance cell migration in a wide range of concentration.

Divergent actions of Pg and MPA have been previously shown using human umbilical vein endothelial cells (HUVECs) (Simoncini *et al.* 2004). In contrast, having in mind that atherosclerosis mainly affect arteries, we focused our attention on the progestins vascular actions on aortic ECs.

Endothelial NO bioavailability plays a pivotal role in the maintenance of vascular homeostasis. Impairment of NO production seriously compromises vascular health and represents a risk factor

for disease development (Duckles & Miller 2010). We showed that, Pg and MPA have opposite effects on NO production in ECs. Physiological concentrations of Pg rapidly stimulate, whereas MPA inhibits NO production. The blockage of progestin's effects with L-NAME confirms that Pg and MPA are affecting NOS derived NO. Evidence reported in the literature shows that Pg increases NO synthesis in human ECs by transcriptional and non-transcriptional mechanisms, whereas MPA lacks such actions (Simoncini *et al.* 2006). Furthermore, chronic administration of Pg to ovariectomized rats restores the endothelial control of vascular tone in mesenteric arterial rings through the endothelial formation of NO, meanwhile MPA failed to do that (Chataigneau *et al.* 2004). On this line of evidence, we found that in ECs isolated from rats devoid of ovarian activity, MPA maintained its inhibitory action on NOS synthesis.

It is known that blood vessels express functional PgR (Perrot-Applanat *et al.* 1988), which would be regulated by Pg as well as by synthetic progestins, like MPA (Sitruk-Ware 2000). The involvement of PgR on cellular and molecular effects of MPA and Pg at the vascular level has been reported. In HUVECs, the PgR antagonist RU486 caused reduction of the inhibitory action of MPA on the E2-induced endothelial NOS (eNOS) phosphorylation and eNOS activity (Oishi *et al.* 2011). In HUVECs, Pg and MPA decreased thromboxane A2 release, and enhanced prostacyclin synthase gene and protein expression in a PgR-dependent manner (Oviedo *et al.* 2011). MPA antagonizes the E2-stimulation of vascular endothelial growth factor (VEGF) expression in human endometrial stromal cells through a mechanism of action that involves the PgR (Okada *et al.* 2011). Using RU486 as PgR antagonist, we obtained evidence that PgR participates in the fast action of both steroids on NO synthesis regulation, with slight differences. The decrease in NO production induced by MPA was completely abolished by the RU486 compound. However, the enhancement in the vasoactive release elicited by Pg was partially dependent on PgR. Although this evidence suggests the participation of PgR on opposite effect on NO production, it remains to determine whether the steroids effects involve the isoforms A or B of PgR, as well as the possible participation of a membrane-anchored receptor, which has been recently described (Zhu *et al.* 2003).

The activity of eNOS is regulated by changes in intracellular calcium and phosphorylation. After agonist stimulation, the enhancement in cytosolic calcium leads to calmodulin binding to eNOS and

subsequent phosphorylation. The enzymes responsible for phosphorylation status depend on the primary stimulus. Several intracellular signaling systems, such as AC/PKA, PLC/PKC, PI3K/Akt, CaMKII, and MAPK, have been involved in the hormonal regulation of eNOS activity (Forstermann 2010). Indeed, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) also participate (Ladurner *et al.* 2012). Employing selective kinase inhibitors we found that the mechanism of action displayed by Pg and MPA on the regulation of endothelial NO production was dependent on MAPK pathway, and independent of PKC activation. Indeed, PI3K was partially involved in Pg action; meanwhile the presence of LY294002 not only suppressed the inhibitory effect of MPA but also enhanced the NO synthesis with respect to control. In hippocampal neurons, both Pg and MPA activate p44/p42MAPK (ERK) signaling pathway, Pg quickly and transiently activates nuclear ERK, while MPA-activated ERK remains cytosolic without nuclear signal (Nilsen & Brinton 2003). MPA induces cell proliferation through up-regulation of cyclin D1 expression via PI3K/Akt/NF-kB cascade in human breast cancer cells (Saitoh *et al.* 2005), or drives PgR to interact with tyrosine kinase c-Src and to activate PI3K signaling pathway, leading to the activation of RhoA/ROCK-2 signaling system (Fu *et al.* 2008b). In human ECs, it has been reported divergent effects of MPA and Pg with a patent difference in the recruitment of MAPK and PI3K signal transduction pathways (Simoncini *et al.* 2006). Taking in account that we have previously reported that Pg stimulates COX activity, we also tested the feasible participation of COX system on Pg and MPA action on NO production. Cross talk between NOS and COX systems has been proposed. Although there is a plethora of evidence in the literature suggesting that increased NO stimulates COX-2, the interaction between NO and prostaglandin is not unidirectional and upregulation of NOS activity by COX has been also reported (Kim 2011; Salvemini 1997). In rat Kupffer cells and in platelets, indomethacin significantly reduced NOS activity (Gaillard *et al.* 1992; Chen *et al.* 1997). Our data suggest that the same intracellular pathways are implicated in opposite effects on NO synthesis elicited by each progestin. Based on the results obtained, a possible explanation for the mechanism of action involved would be that, MAPK and PI3K regulate NOS activity directly or through the modulation of COX activity. However, the results presented in this work cannot establish the sequence of the signal transduction pathways activation. It has been reported that in HUVECs and in brain microvascular ECs, PI3K/Akt and MAPK pathways are involved in COX

expression and activation (Garonna *et al.* 2011; Hsieh *et al.* 2012). Indeed, in HUVECs cells MPA stimulates COX activity (Hermenegildo *et al.* 2005). Having in mind the complexity of the intracellular signaling system, with multiple network interconnections between the different routes, a cross talk between different signal transduction pathways that eventually leads to the activation or inhibition of eNOS may occur. Moreover, considering that we have previously demonstrated that PP1 and PP2A participate in the non genomic action of Pg in rat aortic rings (Mendiberri *et al.* 2006; Cutini *et al.* 2009), the possible participation of phosphatase pathways should not be ruled out. Further experiments are required to fully understand the signaling pathways involved in progestins NOS regulation.

When the effect of the progestins on platelet-ECs interaction was evaluated, we found that Pg exerts an inhibitory effect on platelet adhesion to the endothelium, and in turn prevents the adhesion induced by an inflammatory environment (presence of LPS). Pg also exerts a potent antiaggregatory effect. Thus, the action of Pg may be considered a beneficial fact that contributes to avoid the earlier stage of vascular injury. On the other hand, we suggest that the synthetic progestin is associated with a potentially pro-atherogenic effect, since MPA stimulates platelet adhesion and also potentiates the effect exerted by the presence of LPS, and exhibited a slight antiaggregatory action compared to Pg. These results represent the first evidence of a direct action of MPA on platelet-ECs interactions. The data obtained in platelet aggregation assays suggest that the progestins indirectly modulate platelet aggregation through each direct action on ECs, probably due to the regulation of ECs release of proaggregatory or antiaggregatory vasoactives, such as thromboxane; NO, prostacyclin, that modulate platelet function. The findings that the mechanism of action elicited by each progestin involves NOS and COX transduction systems may support this interpretation. Platelet and monocyte adhesion to endothelium are events modulated through the expression of CAMs. It has been reported that in HUVECs, Pg prevents the expression of the protein and mRNA of TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1), but MPA does not reverse the action of TNF-α (Otsuki *et al.* 2001). In peripheral and cerebral vasculature, MPA causes endothelial disruption, accumulation of monocytes in the vessel wall, and platelet activation, meanwhile, Pg does not show such toxicity (Thomas *et al.* 2003).

ECs proliferation and migration contribute to the development of new capillaries, tissue remodelling and repair. Using wound healing assays we demonstrated that Pg and MPA stimulate ECs migration to the denuded area, in a wide concentration range. In agreement with our results, Fu et al. showed that Pg and MPA enhance ECs migration via the actin-binding protein moesin (Fu *et al.* 2008a). Moreover, Pg promotes ECs movement via the rapid regulation of the focal adhesion kinase (FAK) (Zheng *et al.* 2012). In breast cancer Pg and MPA promote cell movement via rapid actin cytoskeleton remodelling, which are mediated by moesin activation, events triggered by the extra-cellular small GTPase RhoA/Rho-associated kinase (ROCK-2) cascade through partially differing pathways by both steroids (Fu *et al.* 2008b).

Link between NO production and cell migration has been reported. NO stimulates human neural progenitor cell migration via cGMP-mediated signal transduction (Tegenge et al. 2011). Moreover, NO is critically involved in human ECs migration (Schwalm et al. 2010). In contrast, plasma β_2-glycoprotein I inhibits ECs migration through eNOS activation (Chiu et al. 2012). Although MPA and Pg have opposite effects on NO generation, they exhibit similar effects on ECs migration. The explanation for this dual effect remains to be established.

Summing up, the results discussed in this paper contribute to provide information for the comprehension of Pg and MPA regulation of relevant cellular and molecular events of vascular homeostasis. Although it has been assumed that some synthetic progestins used in HRT protocols exert similar clinical actions than natural Pg, both progestogens elicit specific cellular effects. The different pharmacokinetics of natural or synthetic progestins and their diverse affinities for the PgR may lead to the recruitment of partially divergent signaling pathways due to differential modulation of the receptor. The evidence reported to date on the true role of natural Pg and its potential clinical utility is insufficient and unravelling its mechanism of action remains a challenge. Further investigations are required for a complete understanding of the similarities or divergences of natural and synthetic progestins vascular actions.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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649

650

651 **FIGURE LEGENDS**

652

653 **Figure 1**

654 **Effect of Pg and MPA on NO production in ECs.** Starved ECs **(A)** and senile ECs **(B)** were
655 preincubated 30 minutes in the absence or presence of 10 μM L-NAME, and then treated with Pg
656 or MPA at the indicated concentrations for 7 minutes. NO production was measured by Griess
657 reaction as described in Materials and methods section. Results represent the average ± SD of
658 three independent experiments (n=4). (C= control). *P<0.02 vs control.

659

660 **Figure 2. Characterization of ECs derived from young and senile rats.**
661 Microphotographs show representative fields of ECs obtained from young or adult (senile) rats
662 immunostained with anti-CD34 (1 and 2); anti-VEGFR2 (3 and 4) or stained with hematoxylin and
663 eosin (5 and 6). Cell nuclei were counterstained with DAPI (1-4). Details are described in Materials
664 and methods section. Magnification: 600X (1-4) and 100X (5 and 6). The scale bar in 1-4
665 represents 25 μm. The scale bar in 5 and 6 represents 100 μm.

666

667 **Figure 3**

668 **Effect of a PgR antagonist on the regulation by Pg and MPA of NO production.** Starved ECs
669 were preincubated 30 minutes in the absence or presence of 1 μM RU486 or 1 μM RU486 plus 10
670 μM L-NAME, and then exposed to Pg or MPA at the indicated concentrations for 7 minutes. NO
671 production was measured by Griess reaction as described in Materials and methods section.
672 Results represent the average ± SD of three independent experiments (n=4). (C= control). *P<0.02
673 vs control plus vehicle; §P<0.01 vs each Pg; ‡P<0.05 vs control + RU486, †P<0.01 vs each MPA.

674

675 **Figure 4**

676 **Involvement of MAPK, PI3K, and PKC signaling pathways on the regulation of NO**
677 **production by Pg and MPA.** Starved ECs were preincubated 30 minutes in the absence or
678 presence of 5 μM PD98059, 1 μM LY294002, 1 μM chelerythrine, 10 μM L-NAME, and then
679 exposed to 10 nM Pg **(A)** or 10 nM MPA **(B)** for 7 minutes. NO production was measured by Griess

reaction as described in Materials and methods section. Results represent the average ± SD of three independent experiments (n=4). (C= control). *P<0.02 vs control; §P<0.01 vs Pg; †P<0.01 vs control + LY294002; ¶P<0.02 vs control + chelerythrine; ‡P<0.01 vs MPA.

Figure 5

Involvement of COX signaling pathway on the regulation of NO production by Pg and MPA. Starved ECs were preincubated 30 minutes in the absence or presence of 10 μM indomethacin (INDO), and then exposed to 10 nM Pg **(A)** or 10 nM MPA **(B)** for 7 minutes. NO production was measured by Griess reaction as described in Materials and methods section. Results represent the average ± SD of three independent experiments (n=4). (C= control). *P<0.02 vs control; †P<0.01 vs Pg; §P<0.01 vs control plus indomethacin; ‡P<0.01 vs MPA.

Figure 6

Effect of Pg and MPA on platelet adhesion to ECs. ECs cultures were treated with Pg **(A)** or MPA **(B)** at the indicated concentrations for 24 h in the presence or absence of 1 μg/mL LPS, which was added during the last 21 h of hormonal treatment. Platelets were seeded on ECs monolayer for 2 h. Immediately after, the supernatant was removed and the platelets were counted as described in Materials and methods section. Bars represent the means ± SD of the number of adhered platelets/μL. Results are the average ± SD of three independent experiments (n=4). *P<0.05 vs control; **P<0.001 vs control; †P<0.005 vs LPS; ‡P<0.02 vs LPS; §P<0.001 vs each MPA.

Figure 7

Effect of Pg on ECs migration. Confluent ECs cultures were serum starved for 24 h, and cells were removed by scraping. Detached cells were washed with PBS, and the remaining monolayer was treated during 48 h with Pg at the indicated concentrations or vehicle (control). Dotted lines indicate the boundary between the unscratched and scratched areas. (A) Images show representative fields of each condition after Giemsa staining (40X magnification).The scale bar represents 300 μm. (B) Bars show the means and standard deviations of number of migrated

cells/field from three separated experiments performed by quadruplicate. *P<0.001 vs control; §P<0.01 vs control.

Figure 8

Effect of MPA on ECs migration. Confluent ECs cultures were serum starved for 24 h, and cells were removed by scraping. Detached cells were washed with PBS, and the remaining monolayer was treated during 48 h with MPA at the indicated concentrations or vehicle (control). Dotted lines indicate the boundary between the unscratched and scratched areas. (A) Images show representative fields of each condition after Giemsa staining (40X magnification).The scale bar represents 300 μm. (B) Bars show the means and standard deviations of number of migrated cells/field from three separated experiments performed by quadruplicate. *P<0.001 vs control.

Figure 9. Schematic representation of differential regulation of vascular function elicited by Pg and MPA. Pg stimulates while MPA inhibits endothelial NO production. The mechanisms of action involve PI3K, MAPK, and COX signal transduction pathways and PgR participation. The subtype of PgR involved remains to be established. Pg inhibits platelet aggregation and adhesion to CEs, whereas MPA stimulates platelet-ECs interactions. NO generated by the ECs diffuses to the tunica media in order to regulate vascular tone, or may be released to the vascular lumen affecting platelet function. (COX: cyclooxygenase; PgR: progesterone receptor; NO: nitric oxide).

Figure 1

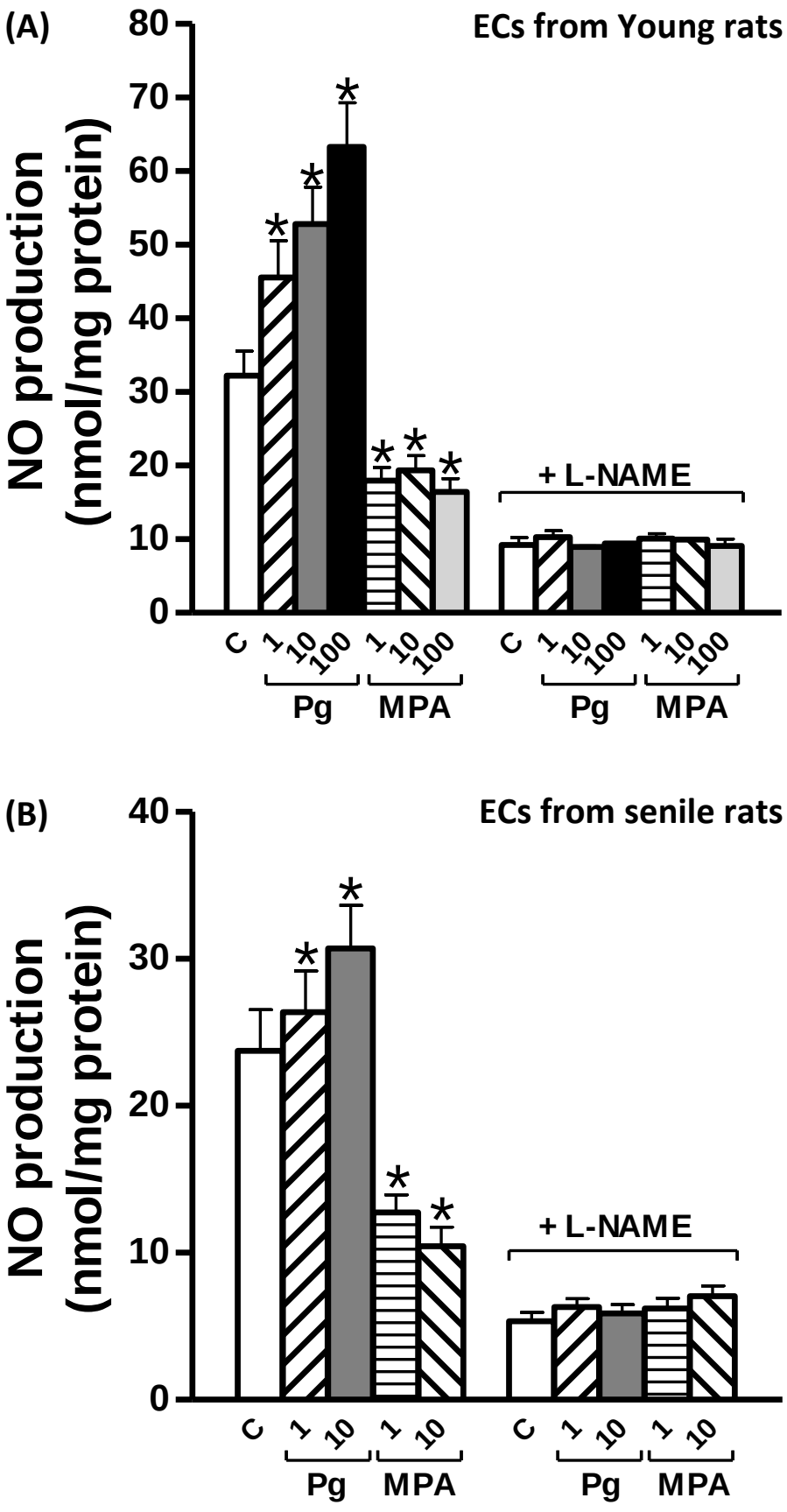

(A)
ECs from Young rats
NO production (nmol/mg protein)
80
70
60
50
40
30
20
10
0
+ L-NAME
C
1
10
100
Pg
MPA
(B)
ECs from senile rats
40
30
20
10
0

Figure 2

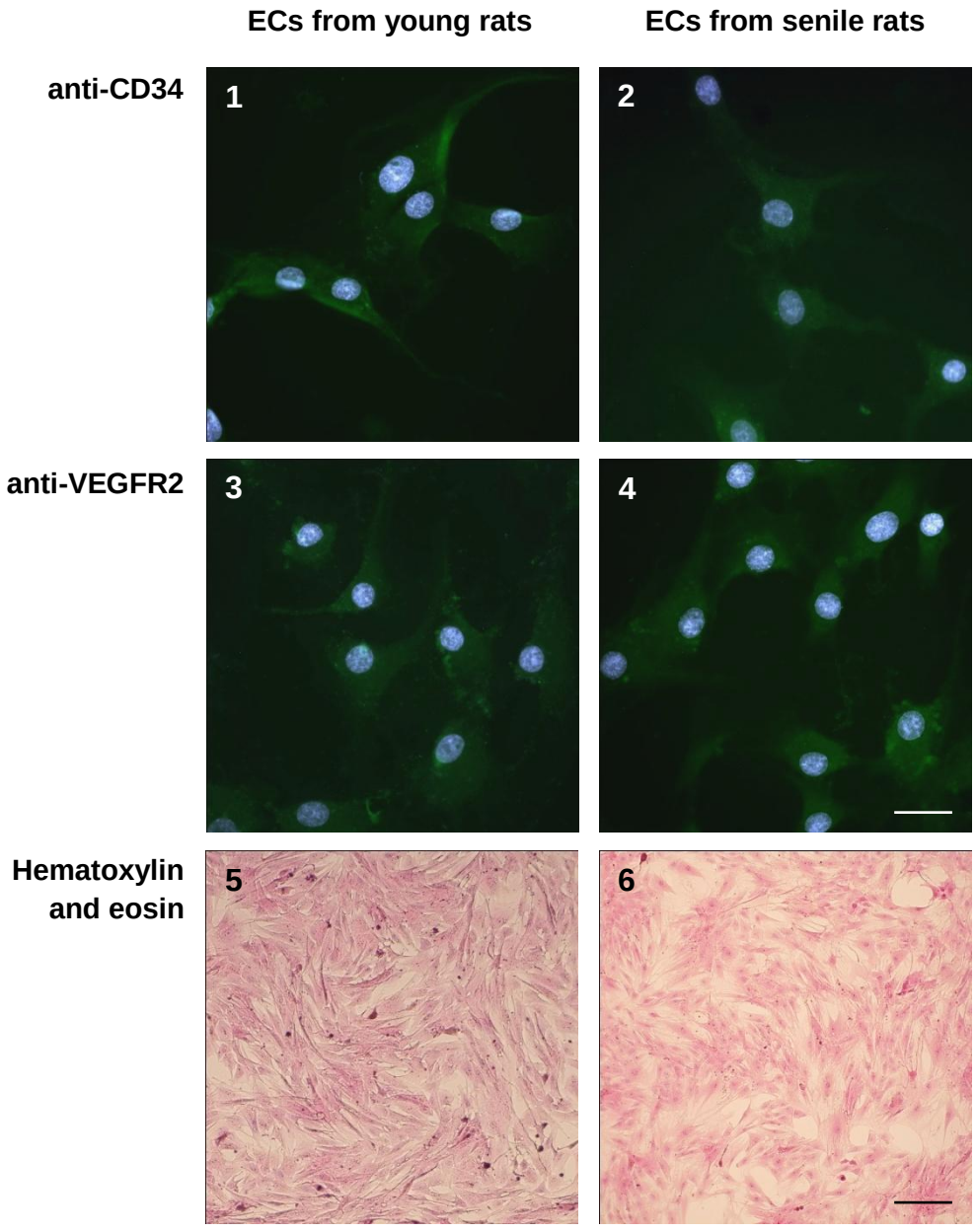

ECs from young rats
ECs from senile rats
anti-CD34
anti-VEGFR2
Hematoxylin and eosin
1
2
3
4
5
6

Figure 3

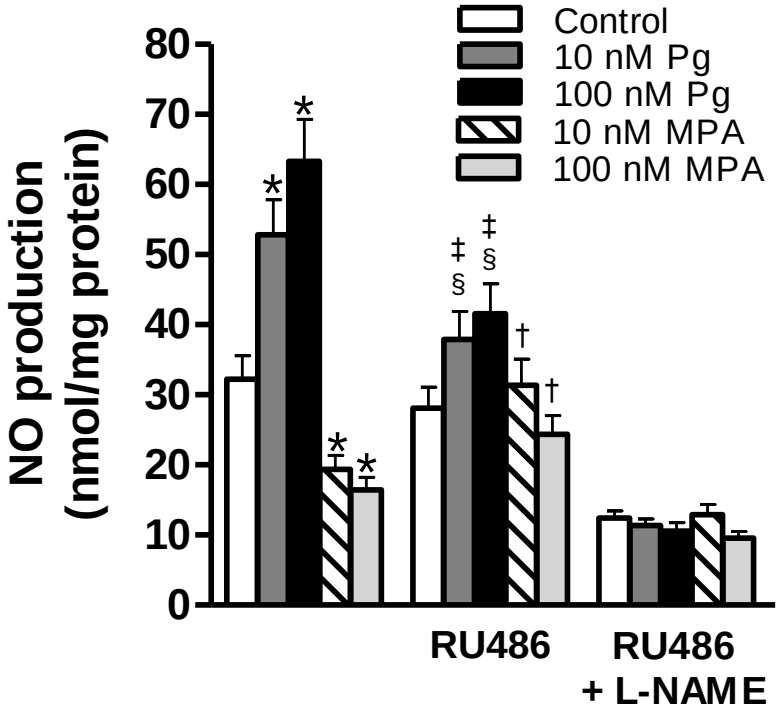

Control
10 nM Pg
100 nM Pg
10 nM MPA
100 nM MPA
NO production
(nmol/mg protein)
80
70
60
50
40
30
20
10
0
*
*
*
*
‡
§
‡
§
†
†
RU486
RU486
+ L-NAME

Figure 4

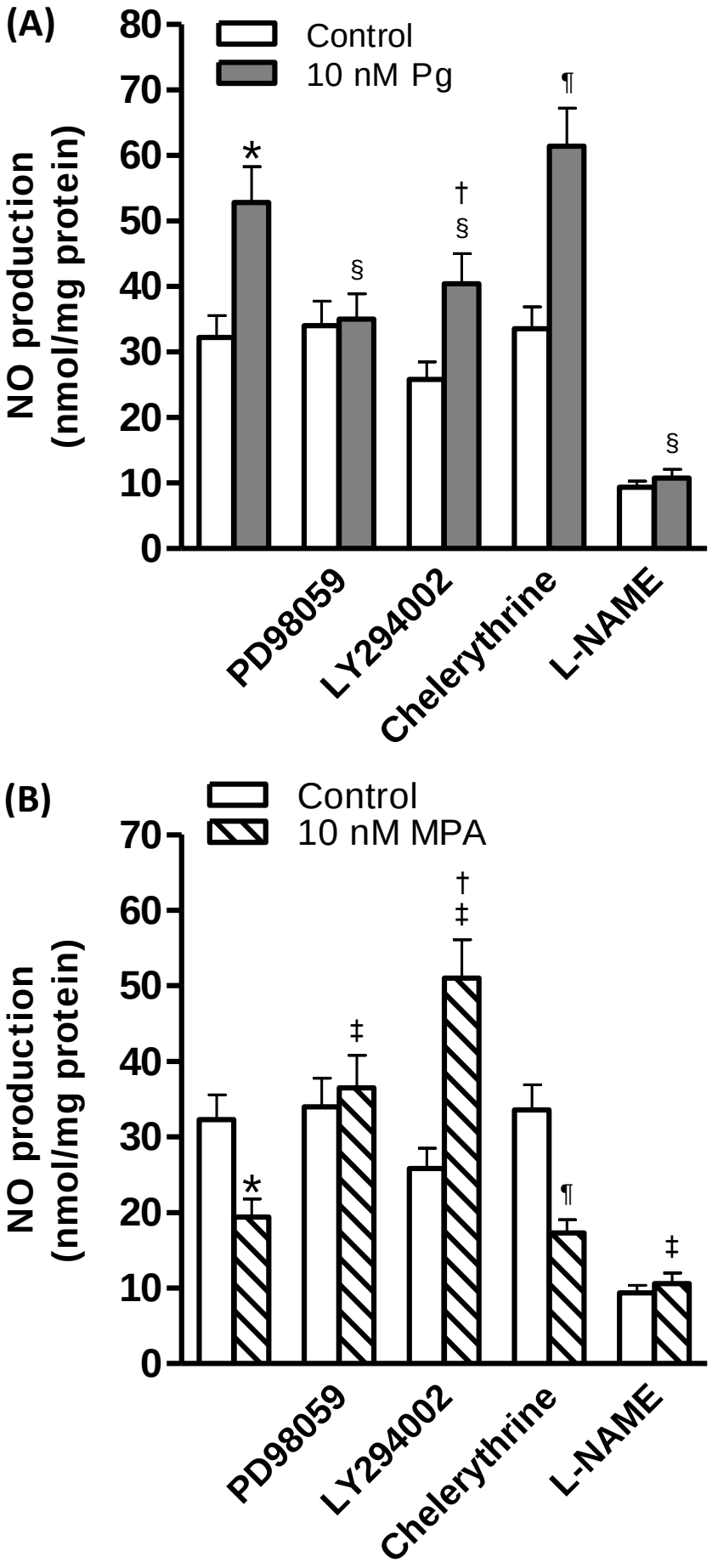

(A)
Control
10 nM Pg
NO production (nmol/mg protein)
0
10
20
30
40
50
60
70
80
*
§
†
§
¶
§
PD98059
LY294002
Chelerythrine
L-NAME
(B)
Control
10 nM MPA
NO production (nmol/mg protein)
0
10
20
30
40
50
60
70
*
‡
†
‡
¶
‡
PD98059
LY294002
Chelerythrine
L-NAME

Figure 5

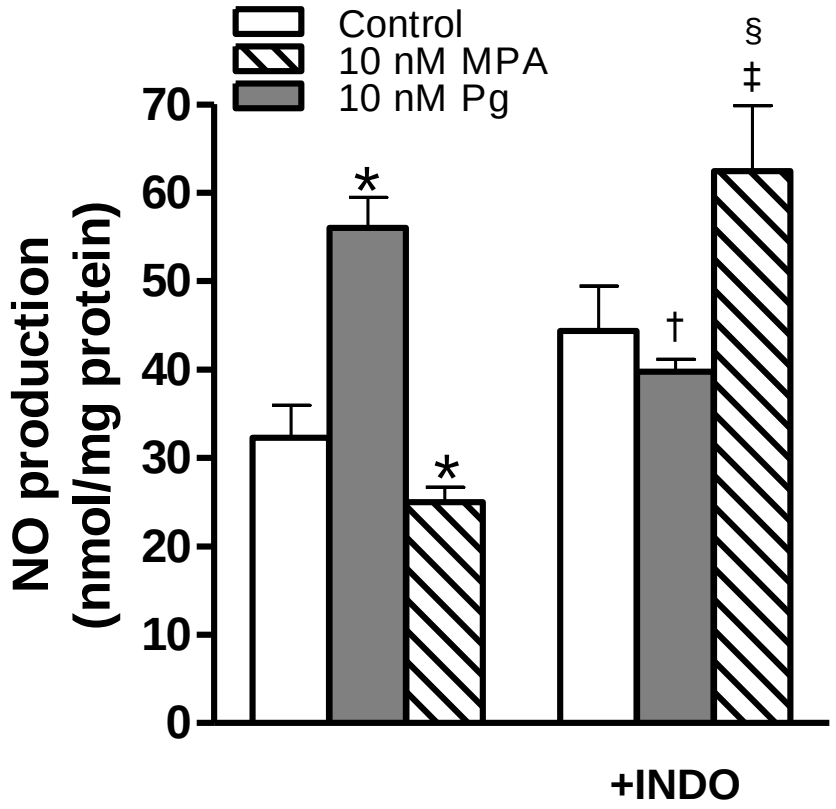

Control
10 nM MPA
10 nM Pg
NO production
(nmol/mg protein)
70
60
50
40
30
20
10
0
*
*
†
§
‡
+INDO

Figure 6

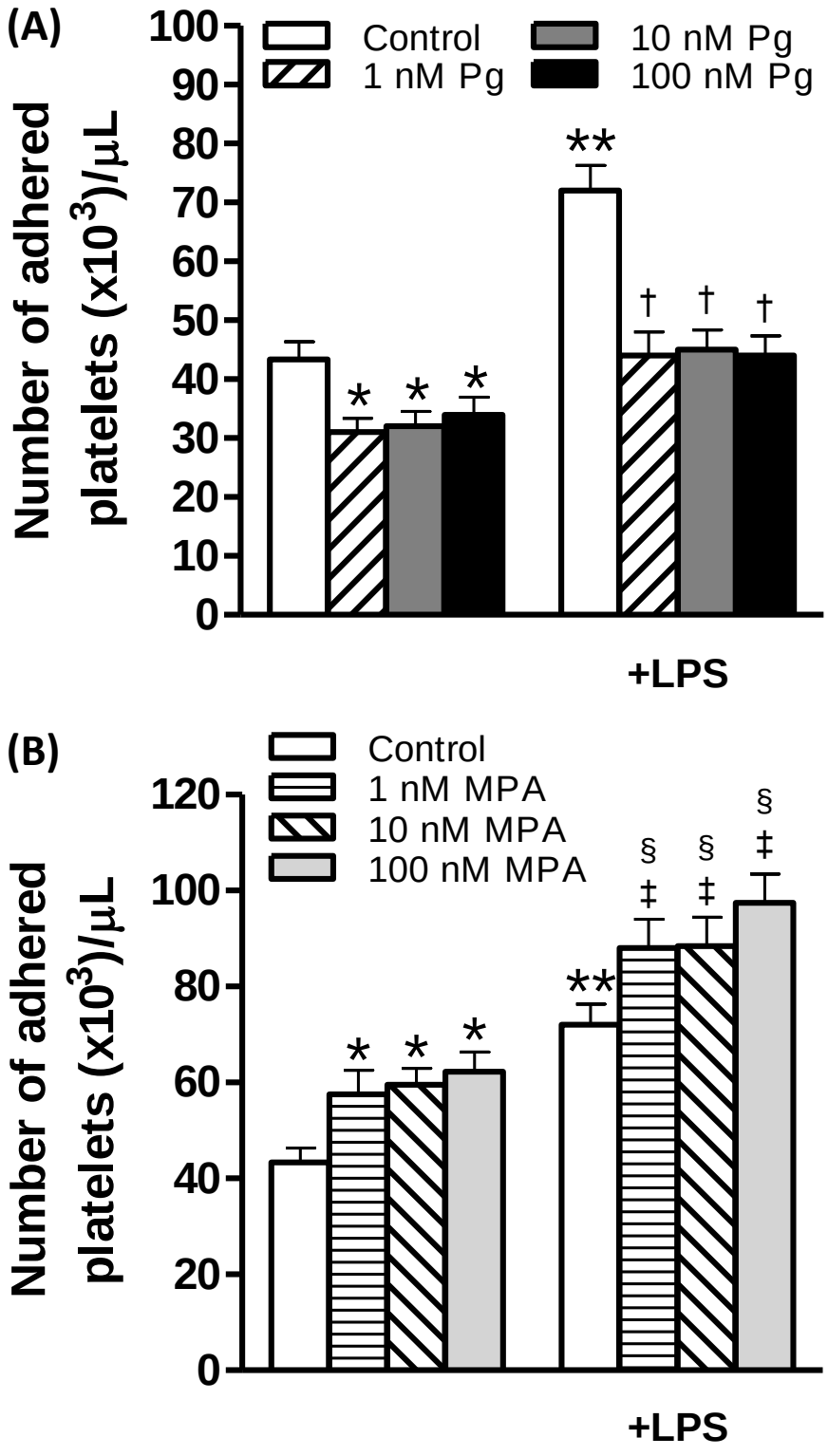
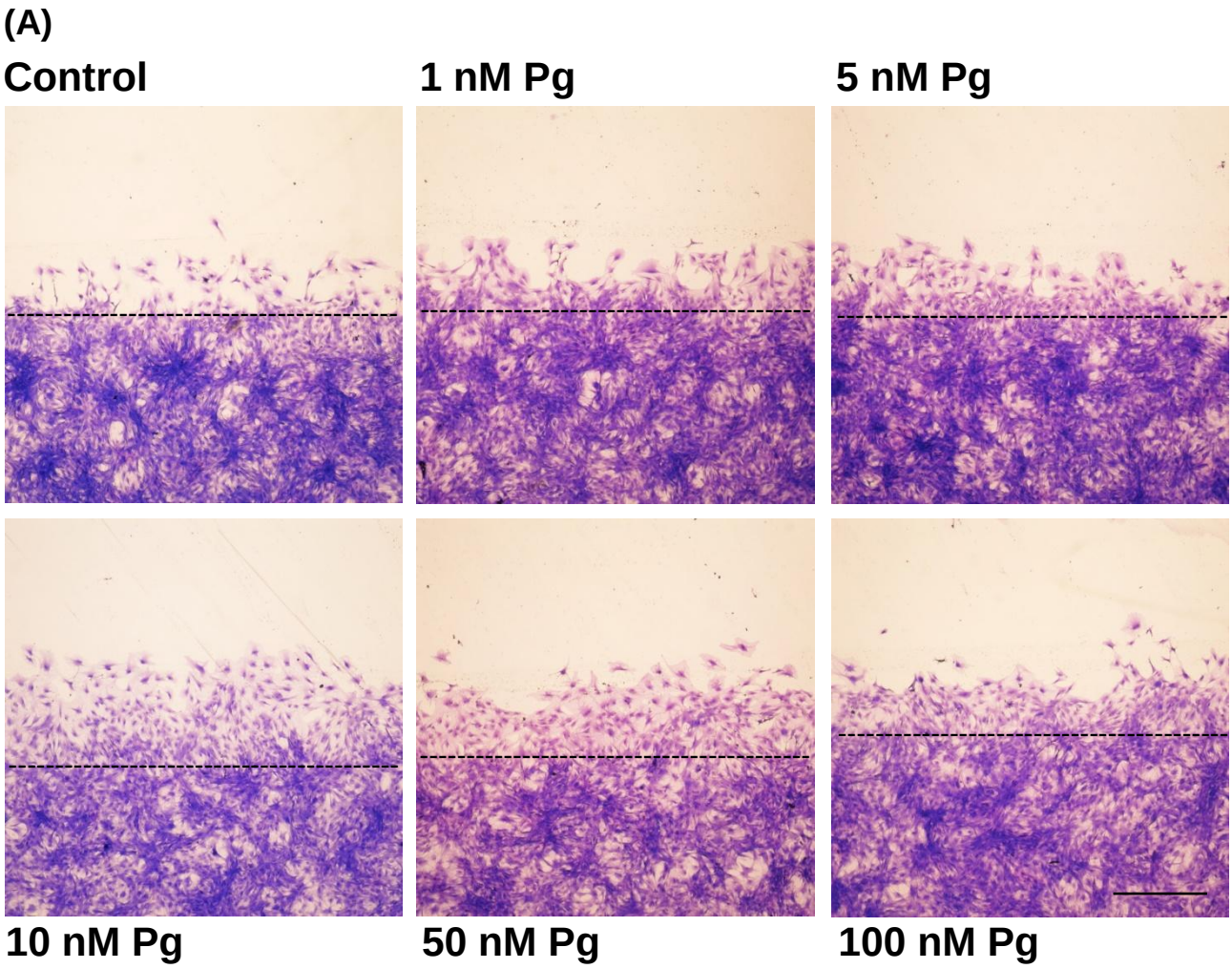

(A)
Number of adhered platelets (x10³)/µL
Control
1 nM Pg
10 nM Pg
100 nM Pg
100
90
80
70
60
50
40
30
20
10
0
*
*
*
**
†
†
†
+LPS
(B)
Number of adhered platelets (x10³)/µL
Control
1 nM MPA
10 nM MPA
100 nM MPA
120
100
80
60
40
20
0
*
*
*
**
§
‡
§
‡
§
‡
+LPS

Figure 7


(A)
Control
1 nM Pg
5 nM Pg
10 nM Pg
50 nM Pg
100 nM Pg

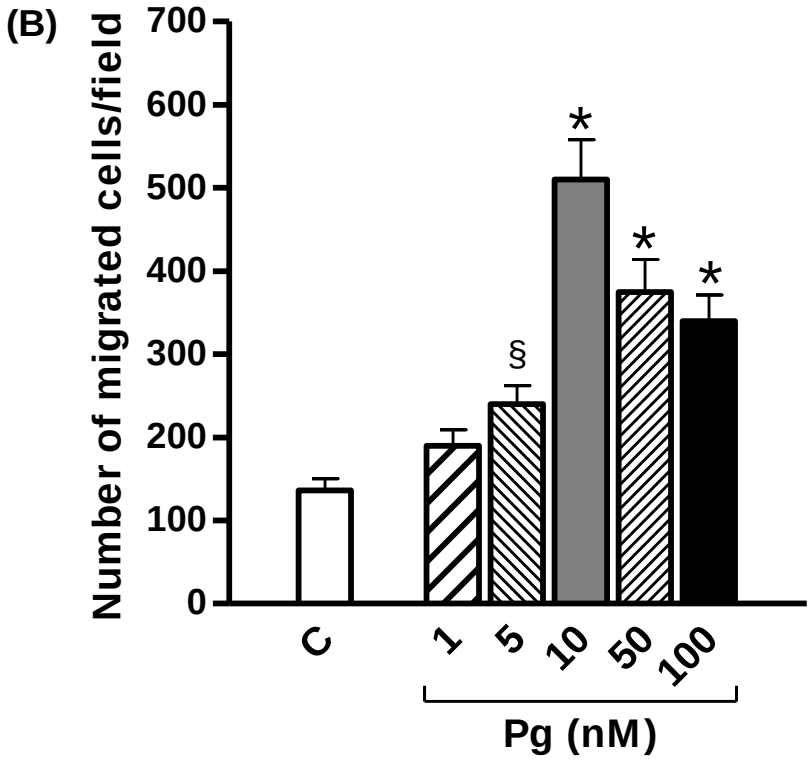

(B)
Number of migrated cells/field
700
600
500
400
300
200
100
0
*
*
*
§
C
1
5
10
50
100
Pg (nM)

Figure 8

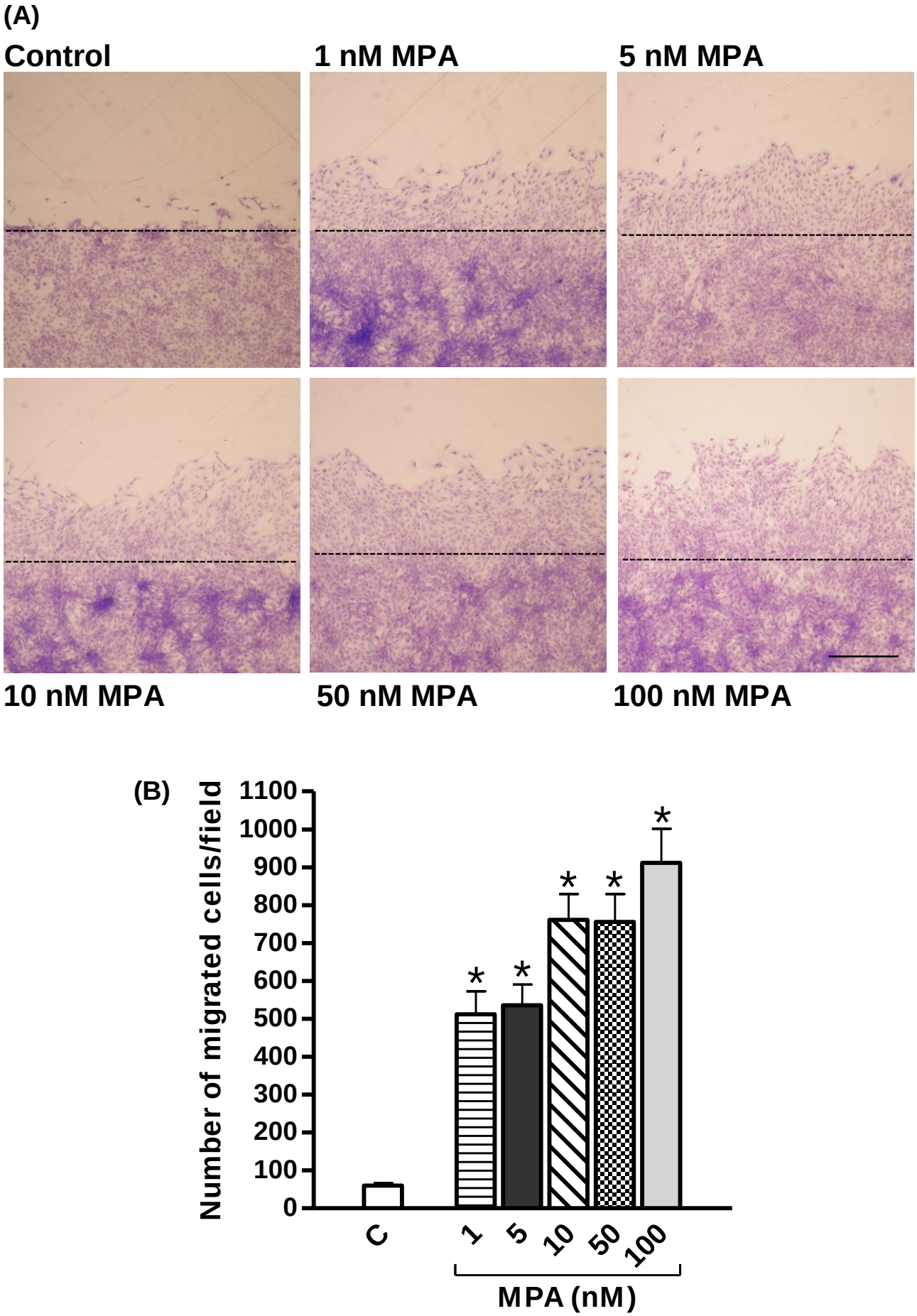

(A)
Control
1 nM MPA
5 nM MPA
10 nM MPA
50 nM MPA
100 nM MPA
(B)
Number of migrated cells/field
1100
1000
900
800
700
600
500
400
300
200
100
0
C
1
5
10
50
100
MPA (nM)
*

Figure 9

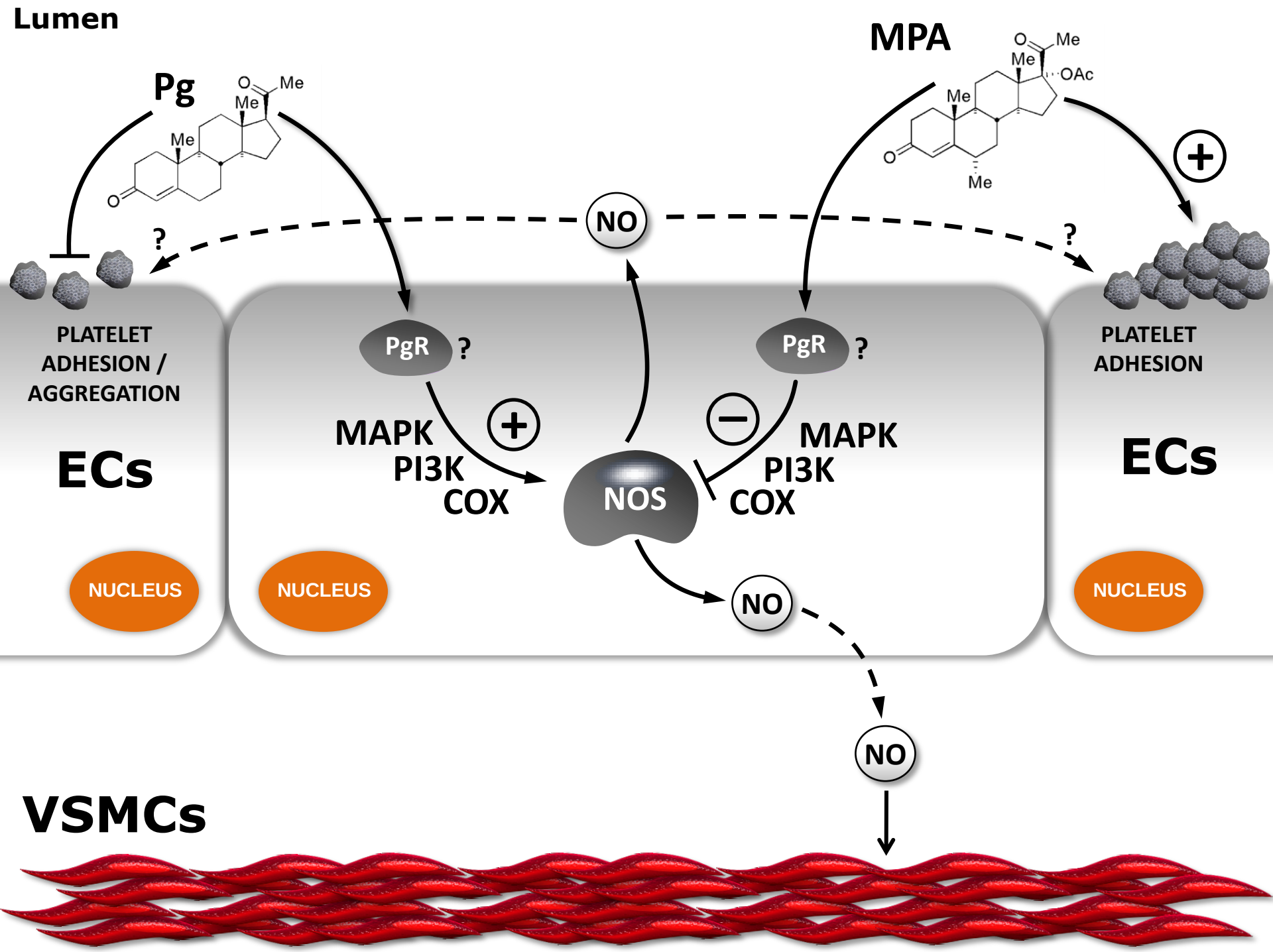

Lumen
Pg
MPA
NO
?
?
PLATELET ADHESION / AGGREGATION
ECs
NUCLEUS
PgR
?
MAPK
PI3K
COX
+
NOS
PgR
?
−
MAPK
PI3K
COX
NO
PLATELET ADHESION
ECs
NUCLEUS
NUCLEUS
NO
NO
VSMCs

Table 1. Effect of Pg and MPA on ECs viability.

Treatment	cell viability (% respect to control)[a]
1 nM Pg	99.8 ± 8.9
10 nM Pg	102.4 ± 7.4
100 nM Pg	90.15 ± 6.3
1 nM MPA	101.8 ± 5.9
10 nM MPA	103.4 ± 5.4
100 nM MPA	98.5 ± 6.3

[a] Subconfluent ECs were incubated in serum-free medium for 24 h and then treated with different concentrations of Pg, MPA or vehicle (control group) for additional 24 h in fresh DMEM containing 1% (v/v) FCS. Cell viability was measured by Crystal Violet assay as described in Material and Methods section. The optical density (OD) value in the control group is considered as 100%. Values are the average ± S.D. of three independent experiments performed by quadruplicate.

Table 2. Effect of Pg and MPA on platelet aggregation.

Treatment	Percentage of inhibition of platelet aggregation with respect to control[a]	Percentage of platelet aggregation with respect to basal[b]
10 nM Pg	82.3 ± 6.2*	97.5 ± 6.5
10 nM MPA	27.2 ± 2.3‡	98.2 ± 7.3

*P<0.01 with respect to control and ‡P<0.05 with respect to control.

[a]Culture medium was replaced by PRP and cells were exposed to 10 nM Pg, 10 nM MPA, or vehicle (control) for 5 minutes. PRP was rapidly removed and platelet aggregation was measured as described in Material and Methods section. Results are expressed as percent of inhibition of platelet aggregation with respect to control group (ECs plus vehicle alone) and are the average ± S.D. of three independent experiments (n=4).

[b]Aliquots of PRP (in the absence of ECs) were incubated with 10 nM Pg or 10 nM MPA for 5 minutes. Basal group received vehicle alone. Immediately after ADP was added, platelet aggregation was measured. Results are expressed as percentage with respect to basal aggregation and represent the average ± S.D. of three independent experiments (n=4).